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Quantitative Analysis of Membrane Remodeling at the Phagocytic Cup[D] [V]

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Nascent phagosomes, which are derived from the plasma membrane, acquire microbicidal properties through multiple fusion and fission events collectively known as maturation. Here we show that remodeling of the phagosomal membrane is apparent even before sealing, particularly when large particles are ingested. Fluorescent probes targeted to the plasma membrane are cleared from the region lining the particle before engulfment is completed. Extensive clearance was noted for components of the inner as well as outer monolayer of the plasmalemma. Segregation of lipid microdomains was ruled out as the mechanism underlying membrane remodeling, because markers residing in rafts and those that are excluded were similarly depleted. Selective endocytosis was also ruled out. Instead, several lines of evidence indicate that endomembranes inserted by exocytosis at sites of ingestion displace the original membrane constituents from the base of the phagosomal cup. The Fcγ receptors that trigger phagocytosis remain associated with their ligands. By contrast, Src-family kinases that are the immediate effectors of receptor activation are flushed away from the cup by the incoming membranes. Together with the depletion of phosphoinositides required for signal transduction, the disengagement of receptors from their effectors by bulk membrane remodeling provides a novel means to terminate receptor signaling.

INTRODUCTION

Phagocytosis is a specialized function of the innate immune system that is performed most efficiently by macrophages and neutrophils (so-called professional phagocytes). Engulfment of invading microorganisms, foreign particles, and apoptotic bodies is initiated by engagement of specialized phagocytic receptors. Activation of these receptors triggers polymerization of actin and remodeling of the plasma membrane, leading to the formation of pseudopodia that ultimately grow to encircle and trap the phagocytic target. Although many receptors are capable of mediating this process, the family of Fcγ receptors, which recognize particles opsonized with immunoglobulin G (IgG), remains the best studied. Clustering of Fcγ receptors, induced by binding to multiple opsonic ligands on a particle, leads to phosphorylation of the FcγR immunoreceptor tyrosine-based activation motif (ITAM) by members of the Src family of kinases, followed by recruitment of the kinase Syk. Syk activation in turn initiates a signaling cascade, including activation of phosphatidylinositol 3-kinase (PI 3-kinase) and of the small GTPases Rac and Cdc42, which coordinate actin remodeling (Henry *et al.*, 2004).

Although the importance of cytoskeletal reorganization at the phagocytic cup has long been appreciated, the role of membrane remodeling has received less attention. Until recently, protrusion of membrane pseudopodia at the phagocytic cup was thought to be mainly a passive event, reflecting pressure-induced deformation of the plasma membrane caused by the stimulated polymerization of subjacent actin (Griffin *et al.*, 1975). More recent data, however, suggest that actin polymerization at the phagocytic cup and pseudopod extension can be affected separately, supporting the notion that membrane remodeling is an independent and important component of the phagocytic response (Lowry *et al.*, 1998; Cox *et al.*, 1999). Indeed, it is now appreciated that membrane remodeling during phagocytosis is an active and complex process that involves localized pinocytosis (Botelho *et al.*, 2002), segregation of membrane components into lipid rafts (Kwiatkowska *et al.*, 2003), lateral diffusion of signaling molecules (Henry *et al.*, 2004), and the insertion of endomembranes by focal exocytosis (Bajno *et al.*, 2000; Braun *et al.*, 2004).

It is currently unclear how the combination of these events alters the composition of the plasma membrane, particularly the specialized area immediately below the target particle, known as the phagocytic cup. Of particular interest is the fate of signaling molecules that are required for triggering and subsequently terminating particle engulfment and the associated inflammatory response. We therefore performed a quantitative assessment of membrane remodeling at the phagocytic cup, using markers of specific membrane microdomains. In addition, we analyzed separately the behavior of the inner and outer leaflets of the plasma membrane, using selectively targeted fluorescent probes that enabled us to perform dynamic measurements in live cells. We report the occurrence of marked changes in membrane composition that precede

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completion of phagocytosis, which vary greatly depending on the size of the particle being internalized.

MATERIALS AND METHODS

Reagents and Antibodies

Fetal bovine serum (FBS), DMEM, HEPES-buffered solution RPMI-1640 (HPMI), and phosphate-buffered saline (PBS) were from Wisent (St. Bruno, Quebec, Canada). Alexa Fluor 555–conjugated cholera toxin subunit B (recombinant), Alexa Fluor 633–conjugated transferrin, and FM4-64 were from Molecular Probes (Carlsbad, CA). Latex beads (3.1 or 8.3 μm in diameter) were from Bang's Laboratories (Fishers, IN). Rat anti-LAMP1 antibody (clone ID4B) was from the Developmental Hybridoma Bank. Goat anti-Lyn polyclonal antibody was from R&D Systems (Minneapolis, MN). Cy3-labeled Fab fragment of anti-mouse antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). Human IgG, LY294002, and all other reagents were from Sigma Aldrich Canada (Oakville, Ontario, Canada).

DNA Constructs

The wild-type (WT) *N*-ethylmaleimide sensitive factor (NSF) and dominant-negative (DN) NSF constructs used in this study have both been described elsewhere (Coppolino *et al.*, 2001). Briefly, DN-NSF contained a single codon mutation of glutamate at position 329 to glutamine (E329Q) and was generated using site-directed mutagenesis. The activity of both WT and DN-NSF constructs has been verified in vitro using an ATPase assay and transfection of the constructs is known to result in exogenous NSF levels that are three times higher than the endogenous NSF levels as early as 8 h later (Coppolino *et al.*, 2001). FcγRIIA-CFP and green fluorescent protein (GFP) were constructed by cloning a cDNA of FcγRIIA into pEGFP-N1 (Clontech, Palo Alto, CA) or CFP at HindIII and SacII sites. PM-RFP was generated by digesting RFP with XhoI and BamHI and ligating the cut vector with annealed PM oligonucleotides that had been synthesized with the appropriate sticky ends using a commercial service. The GPI-yellow fluorescent protein (YFP) construct was a gift from Dr. M. Edidin (Johns Hopkins University, Baltimore, MD). YFP-GT46 is a chimeric secretory protein containing a signal sequence fused to YFP, a consensus N-glycosylation site, the transmembrane domain of the LDL receptor, and the cytoplasmic tail of CD46 (Kenworthy *et al.*, 2004). The vectors encoding GFP-Rab5 and DN dynamin I (K44A) have been previously described in Scott *et al.* (2005) and Damke *et al.* (1994), respectively. The plasmid encoding GFP-EEA1(1257-1411) was a gift from Dr. H. Stenmark (Norwegian Radium Hospital, Oslo, Norway). Kinase-dead Lyn (K275R) was a gift from Dr. J. Bolen (Bristol Myers Squibb). GFP-Sec61 was a gift from Dr. J. Brumell (Hospital for Sick Children, Toronto).

Cell Culture and Transfection

RAW264.7 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in DMEM with 5% FBS at 37°C in 5% CO_2 under a humidified atmosphere. Transfections were performed using the Amaxa electroporation system (Gaithersburg, MD) following the manufacturer's guidelines, using 4 million cells and 3 μg of cDNA per coverslip and program U14. Stable lines expressing the GPI-anchored yellow fluorescent protein (YFP-GPI) were selected with G418 (0.5 mg/ml). Cells were cotransfected with DN- or WT-NSF and EGFP (or RFP) in a 9:1 ratio and were analyzed 8–10 h later. Immunofluorescence staining using a polyclonal antibody, generated and kindly provided by Dr. W. Trimble (Hospital for Sick Children, Toronto), was performed to confirm that under these conditions greater than 95% of the EGFP-positive cells ectopically expressed NSF (Coppolino *et al.*, 2001). Where indicated, cells were transfected with plasmids encoding dynamin I (K44A) and EGFP (5:1 ratio) and used between 24 and 48 h later. The effectiveness of the DN dynamin was confirmed by monitoring the uptake of fluorescently labeled transferrin.

Phagocytosis Assays and Endocytosis

Latex beads were opsonized with 3.75 mg/ml human IgG for 1 h at 37°C. Cells grown on glass coverslips were changed to prewarmed serum-free medium and were overlaid with 25 μl of opsonized latex beads and incubated at 37°C to initiate phagocytosis. In most experiments, phagocytosis was synchronized by centrifugation of the cells at 1500 rpm for 1 min after the addition of beads. RAW cells were pretreated with 10 μM colchicine for 10 min or with 100 μM LY 294002 for 15 min before phagocytosis.

Endocytosis at the phagocytic cup was detected by incubating RAW cells with 10 μM FM4-64 for 30 s at 4°C then adding opsonized beads to the cells. Cells were incubated for 2.5 min at 37°C and then washed five times with cold PBS before imaging.

To label FcγRIIA in internal compartments, cells transfected with the GFP-conjugated receptor (see above) were incubated with 300 ng/ml anti-human FcγRIIA monoclonal Fab fragment for 60 min, followed by a Cy3-conjugated secondary Fab fragment, both in the cold to prevent internalization. Cells were next incubated at 37°C for 15 min to internalize the labeled receptor, and the remaining surface label was removed using a brief acid wash, as previously described (Tuma *et al.*, 2002). Finally, opsonized beads were added to the cells for 6 min, at which point the macrophages were fixed in 4% paraformaldehyde and imaged.

Microscopy, Immunofluorescence, and Image Analysis

Analysis of the distribution and density of the membrane markers was performed using an LSM510 laser scanning confocal microscope (Zeiss, Thornwood, NY) with a 100× oil immersion objective. Spectral separation of GFP from YFP was performed using the LSM510 Meta system. Quantitation of fluorescence was done using Image J software (NIH) after subtracting for background.

Detection of LAMP1 in RAW cells by immunofluorescence was performed after fixing cells in 4% paraformaldehyde, followed by permeabilization in 0.1% Triton, blocking in 5% milk, then probing with rat anti-LAMP1 (clone ID4B) at 1:100 for 1 h. After washing with PBS, samples were incubated with Cy3 anti-mouse antibody for 30 min, then washed, and mounted on slides using Dako mounting medium (Carpinteria, CA). Immunofluorescence staining of endogenous Lyn was performed as above, using ice-cold methanol as the fixative and blocking with 5% donkey serum.

Electron Microscopy

RAW cells were preincubated with 2.2 mg/ml cationized ferritin in veronal buffer for 2.5 min at 4°C. The extracellular medium was then changed to fresh HPMI, and opsonized beads were added to the cells, which were then incubated at 37°C for 2.5 min. Cells were then fixed in 2% glutaraldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.2, for 10 min at 4°C, before fixation was continued at room temperature for at least a further 1 h. Cells were then postfixed in 1% OsO_4 in phosphate buffer at room temperature for 2 h, stained en bloc for 1 h with 1% uranyl acetate in H_2O, and then dehydrated and embedded in Epon resin. Sections were viewed using a FEI Tecnai 20 electron microscope (Beaverton, OR), and images were captured using a Gatan Dualview camera (Pleasanton, CA).

Statistics

Unless otherwise stated, all experiments were performed at least in triplicate. For comparisons of means, *t* tests were used. $p < 0.05$ was deemed significant.

RESULTS

Clearance of GPI-anchored Proteins from the Phagocytic Cup

We analyzed the fate of YFP-GPI as a measure of membrane remodeling during formation of the phagocytic cup. We generated a line of RAW264.7 murine macrophages stably transfected with this chimeric construct and observed its distribution before and during phagocytosis using confocal microscopy of live cells. Consistent with earlier findings, YFP-GPI was largely found at the plasma membrane of quiescent RAW cells, with little intracellular fluorescence. On the initiation of phagocytosis of large (8 μm) opsonized latex beads, distinct membrane pseudopods could be observed extending around the particle. Remarkably, there was an obvious clearance of YFP-GPI from the base of the phagocytic cup within minutes of particle contact, even before the pseudopods had fully surrounded the particle (Figure 1A). The fluorescence at the base of the cup dropped to 49 $\pm$ 4% of that of the plasma membrane within 5 min after initiation of phagocytosis (mean $\pm$ SE of 30 determinations). This pronounced and highly localized loss of fluorescence at the base of the cup was not due to an optical artifact, as the distribution of fluorescently labeled opsonin around the surface of the latex bead was homogeneous, even in the area where YFP-GPI fluorescence was greatly reduced (Figure 1B).

Endocytosis Occurs at the Phagocytic Cup, But Does Not Account for Clearance of YFP-GPI

The clearance of GPI-anchored proteins from the base of the phagocytic cup might be explained by localized endocytosis. Endocytosis at the cup is conceivable because neutrophils, which are also professional phagocytes, were reported to perform active pinocytosis during phagocytosis (Botelho *et al.*, 2002). We devised an assay to detect endocytosis at the

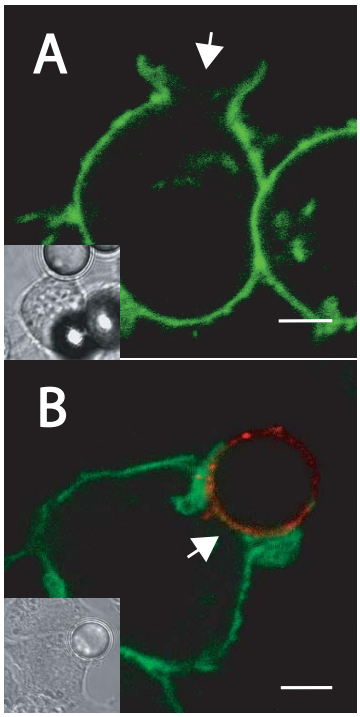

Figure 1. Clearance of YFP-GPI from the base of the phagocytic cup. RAW264.7 macrophages stably transfected with YFP-GPI were allowed to internalize IgG-opsonized latex beads (8-μm diameter) while acquiring confocal fluorescence images. (A) Representative image acquired 1 min after engagement of the bead. (B) To rule out the possibility that the apparent clearance of YFP-GPI (indicated by arrows in A and B) is an optical artifact caused by the distinct refractive index of the bead, opsonized beads were labeled with donkey anti-human Cy3 secondary antibodies before phagocytosis. Despite clearance of YFP-GPI from the base of the phagocytic cup, the opsonin is visible continuously around the bead. For quantitation, see text. Insets show corresponding DIC images. Scale bars, 4 μm. Images are representative of over 30 cells from three experiments.

cup by labeling the plasma membrane of RAW cells with the amphiphilic fluorophore FM4–64. This solvochromic probe selectively exhibits fluorescence when in the hydrophobic environment of the lipid bilayer, yet can be removed from the plasma membrane by simply washing the cells. RAW cells were initially incubated with FM4-64 at 4°C, to label the plasmalemma while preventing endocytosis. This resulted in homogeneous labeling of the plasmalemma (not illustrated). The macrophages were then exposed to opsonized latex beads, allowing binding of the beads to Fcγ receptors. Phagocytosis was then induced by sudden warming to 37°C, followed by termination and washing of extracellular FM4-64 by repeated washing in the cold. Under these conditions, only dye trapped within sealed cellular compartments is retained. As illustrated in Figure 2A, when a brief (2.5 min) incubation at 37°C is performed, most of the fluorescence is detected in a punctate distribution near the base of the forming phagosome, implying that endocytic activity is much greater in this region than elsewhere in the cell.

Two methods were used to determine whether the observed endocytosis could account for the acute disappearance of plasmalemmal markers from the base of the phagosome. First, endocytosis was studied in cells transfected with YFP-GPI (Figure 2B). As before, clearance of YFP-GPI was noted at the base of forming phagosomes and endocytic vesicles loaded with FM4-64 were clearly discernible; however, there was little colocalization of the two probes in endocytic vesicles. The possible contribution of endocytosis was also studied by transfecting the cells with a DN allele of dynamin, a GTPase that is required for both clathrin-dependent and caveolar endocytosis. The effectiveness of this approach was first tested by measuring the uptake of transferrin, which in untransfected cells is internalized via clathrin-mediated endocytosis. As reported earlier (Damke *et al.*, 1994), expression of dynamin I (K44A) prevented the endocytosis of transferrin by RAW cells (not shown). Under these conditions, clearance of the plasma membrane from the base of the phagocytic cup was still observed (Figure 2C). Although the possible existence of dynamin-independent endocytic processes cannot be excluded, these two lines of evidence jointly argue against endocytosis as the primary mechanism underlying the removal of membrane markers from the base of the forming phagosome.

Dynamics of Other Membrane Markers during Phagocytosis

Localized endocytosis of YFP-GPI would explain its clearance from the phagocytic cup only if the internalization process were selective for this lipoprotein, as nonselective endocytosis would reduce membrane area while leaving the net concentration of YFP-GPI unchanged. To further analyze the mechanism of membrane remodeling, we

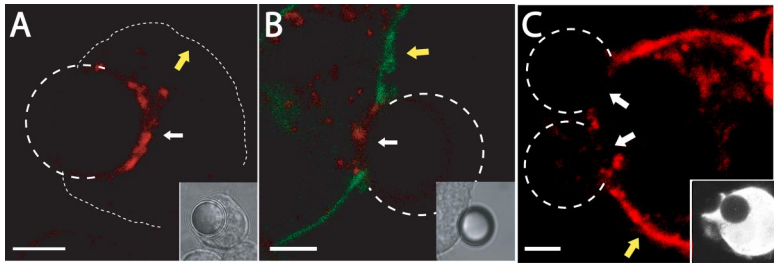

Figure 2. Endocytosis occurs at the phagocytic cup but does not account for clearance of YFP-GPI. The impermeant dye FM4-64 (red) was added to either wild-type (A) or YFP-GPI transfected cells (B). Phagocytosis was initiated by the addition of opsonized latex beads. After 2.5 min the cells were washed repeatedly (five times) to remove any dye that had not been internalized by the cells, and images were acquired immediately. Note that no FM4-64 remains on the plasma membrane (dotted line in A, indicated by yellow arrow in A and B), whereas vesicles labeled by the dye are visible in the vicinity of the phagocytic cup (white arrow in A and B). Note also that most FM4-64–labeled vesicles in B contain little YFP-GPI (shown in green). Insets in A and B show corresponding DIC images. In C cells were transfected with the dominant negative (K44A) form of dynamin I plus soluble GFP (5:1 ratio). After allowing expression of the proteins (24–48 h) the cell membrane was labeled with fluorescent (red) B subunit of cholera toxin. Opsonized particles were then added and fluorescence was monitored by confocal microscopy. White arrows in C denote sites of cup formation, where clearance of the probe was readily apparent, whereas it was retained in the bulk membrane (yellow arrow). The dashed line shows the outline of the beads. The inset in C shows the presence of soluble GFP, used as an indicator of dynamin coexpression. Scale bars, 4 μm. Images are representative of over 30 cells from three experiments.

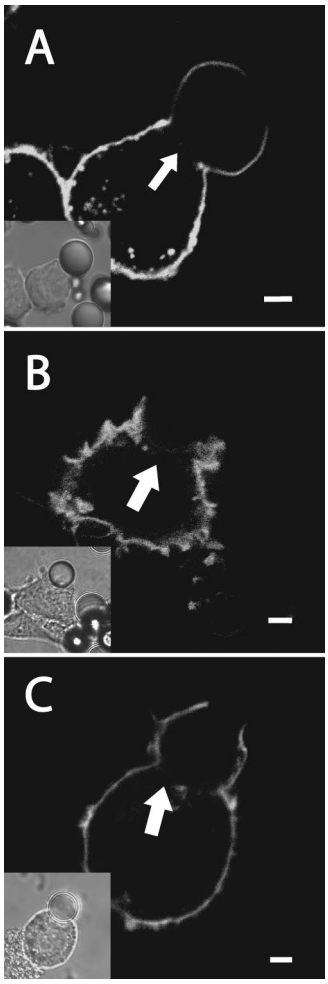

Figure 3. Clearance of membrane markers from the phagocytic cup is not due to lipid raft segregation. RAW macrophages were allowed to ingest 8-μm IgG-opsonized beads, and confocal fluorescence images were acquired 1 min after particle engagement. (A) Cells were labeled with Alexa 555–conjugated cholera toxin B-subunit before phagocytosis, as described in *Materials and Methods*. (B) Cells were transfected with PM-GFP. (C) Cells were transfected with YFP-GT46. Arrows denote areas where clearance of the membrane marker has begun. For quantitation, see text. Insets show corresponding DIC images. Scale bar, 2 μm. Images are representative of over 30 cells from three experiments for each condition.

monitored the fate of other probes as well. RAW cells were labeled with fluorescent cholera toxin B subunit before phagocytosis. This subunit of the toxin binds to GM1 gangliosides and is widely used as a plasma membrane marker. We observed rapid and efficient clearance of fluorescent B subunit from the base of the phagocytic cup, even before sealing of the phagosome was completed (Figure 3A). In three similar experiments, only 31 $\pm$ 5% of the original plasma membrane fluorescence remained at the cup within 5 min after initiation of phagocytosis. This finding implies that the disappearance from the cup is not restricted to YFP-GPI. Of note, both GPI-tagged proteins and GM1 gangliosides reside in the outer leaflet of the plasmalemma. We therefore transfected RAW cells with a marker of the inner leaflet, plasma membrane GFP (PM-GFP; Teruel *et al.*, 1999), to ensure that the phenomenon was not limited to the outer leaflet. PM-GFP, a diacylated probe containing the N-terminal domain of Lyn, behaved in a manner similar to that described above for the exofacial probes, becoming depleted from the base of the phagocytic cup before the bead was completely internalized (Figure 3B).

Segregation of Lipid Microdomains Fails To Account for Membrane Remodeling in Phagocytosis

We next considered the possibility that the membrane remodeling we observed might reflect lateral segregation of lipid rafts, microdomains of the plasma membrane enriched in cholesterol and sphingolipids. A number of transmembrane receptors are preferentially associated with lipid rafts, which have been postulated to serve as scaffolds on which signaling molecules assemble (Razzaq *et al.*, 2004). The three probes used above, GPI-linked YFP, cholera toxin bound to GM1 gangliosides, and PM-GFP, are all believed to reside predominantly in lipid rafts. It was therefore conceivable that exclusion of rafts from sites of phagocytosis might account for the pronounced clearance we observed. To address this possibility, we transfected RAW cells with YFP-GT46, a chimeric transmembrane protein that does not reside in lipid rafts (Kenworthy *et al.*, 2004). Macrophages expressing YFP-GT46 exhibited fluorescence mostly at the plasma membrane. The density of YFP-GT46 at the base of the cup diminished drastically during the early stages of phagocytosis (Figure 3C), as described for the other probes. In three similar experiments, 55 $\pm$ 7% of the original plasma membrane fluorescence remained at the cup within 5 min after initiation of phagocytosis.

Taken together, these observations indicate that clearance of membrane markers at the phagocytic cup cannot be accounted for by either selective endocytosis or by lateral segregation of lipid domains.

Clearance of Membrane Markers Is due to Exocytosis

The extensive and rapid clearance of fluorescent membrane markers from the base of forming phagosomes could result from the focal insertion of unlabeled endomembranes. To test this hypothesis we performed transmission electron microscopy (TEM) of RAW cells that had been briefly pulsed with cationized ferritin. Under these conditions, cationized ferritin binds diffusely to the plasma membrane, where it can be readily detected as an electron-dense coating by TEM. After removal of unbound ferritin from the medium, the cells were exposed to opsonized beads and then fixed and processed for TEM after $\approx$2 min, at a stage where the phagocytic cup has formed but not yet sealed around the bead. Although the membrane of quiescent cells was labeled continuously and rather homogeneously throughout its surface (not shown), a drastic reduction of nearly 50% in the density of ferritin was noted in the surface membrane in the immediate vicinity of the phagocytic target, whereas a 13% drop was noted on the membrane lining the outer face of the pseudopods (Figure 4, A–D). Importantly, ferritin-bearing endosomes were only rarely seen near the cup, suggesting that depletion of the electron-dense probe is not caused by selective internalization, which is consistent with the conclusion reached using fluorescent probes. Instead, the data suggest that ferritin and other components of the plasma membrane are displaced laterally from the base of the cup by unlabeled endomembranes that are delivered focally by directed exocytosis. The resulting dilution would account for the decreased density of both electron-dense and fluorescent probes observed.

Recent work has implicated microtubules in the delivery of endomembrane vesicles to the phagocytic cup (Tapper *et al.*, 2002; Nishida *et al.*, 2005). This enabled us to test

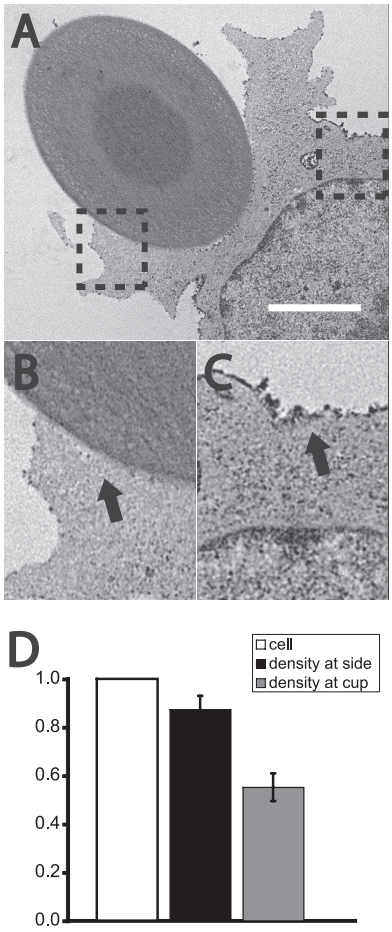

Figure 4. Insertion of endomembranes by exocytosis detected by electron microscopy. RAW macrophages were labeled with cationized ferritin (see *Materials and Methods*) before exposure to 8-μm opsonized beads. After 2.5 min, the cells were fixed and subjected to conventional transmission electron microscopy. The areas boxed by the dotted lines in A are magnified in B (lower left box) and C (upper right box). (B) A portion of the pseudopod; (C) a region of the unengaged membrane. In D the density of ferritin at the cup and on the membrane lining the side of the pseudopods not in direct contact with the particle was quantified and normalized to that in the bulk, unengaged plasma membrane. Data are means ± SE densities from nine different determinations. Note the different density of ferritin in the regions indicated by the arrows. Scale bar, 2 μm. Micrographs are representative of three experiments.

experimentally the contribution of exocytosis to membrane remodeling during phagocytosis. When RAW macrophages stably expressing YFP-GPI were pretreated with colchicine, binding of opsonized beads proceeded normally and phagocytic cups were formed. Clearance of YFP-GPI from the base of such cups, however, was essentially ablated (Figure 5, A and B); the fluorescence at the cup was indistinguishable from that elsewhere on the cell surface. The density of YFP-GPI at the cup relative to that in unengaged regions of the membrane was significantly lower in control cells than in colchicine-treated ones ($p < 0.01$; Figure 5B).

These data are consistent with the premise that localized exocytosis contributes to the dilution of plasmalemmal components at the cup. Additional support for this notion was obtained using LY294002, a specific and potent inhibitor of PI 3-kinase. Others have demonstrated that blockade of this enzyme arrests phagocytosis of large (>3 μm) particles at the cup stage without preventing actin polymerization, suggesting that it is important for membrane delivery to the cup (Cox *et al.*, 1999). As expected, YFP-GPI–transfected macrophages pretreated with LY294002 were largely unable to internalize 8-μm beads, despite forming distinct phagocytic cups. Clearance of YFP-GPI from the phagocytic cup was virtually eliminated (Figure 5, C and D). The difference in marker density at the cup between control and LY294002-treated cells was highly significant ($p < 0.001$).

Membrane fusion events, including exocytosis, are known to involve the soluble-NSF attachment protein receptor (SNAREs) protein family. SNARE proteins on the vesicular and target membranes form complexes that are thought to bring the membranes into close apposition, leading to fusion. Hexameric ATPase NSF is required for the uncoupling and repriming of these complexes, and inhibition of NSF has been shown to block exocytosis (Coppolino *et al.*, 2001). To further document the role of exocytosis in phagosomal remodeling, we transfected YFP-GPI–expressing macrophages with either DN-NSF or WT-NSF. Cells (over)expressing WT-NSF exhibited normal phagocytic cup formation, with clearance of YFP-GPI from the base of the cup that was indistinguishable from untransfected cells (Figure 5F). In contrast, cells expressing DN-NSF formed phagocytic cups but were unable to clear the membrane marker (Figure 5, E and F). The differential behavior of cells transfected with WT- and DN-NSF was statistically significant ($p < 0.05$). Taken together, these data demonstrate that clearance of the membrane marker at the phagocytic cup is due largely to focal exocytosis.

Membrane Remodelling Is Size-dependent

As alluded to earlier, inhibition of PI 3-kinase arrests phagocytosis while the phagocytic cup is still unsealed. This effect is much greater for large particles (>3 μm) than for smaller ones and has been attributed to a requirement for PI 3-kinase in exocytosis (Cox *et al.*, 1999). This implies that the contribution of exocytosis to the formation of the phagocytic cup is different for different-sized particles. To test this hypothesis, RAW macrophages were transfected with either YFP-GT46 or YFP-GPI and allowed to ingest opsonized 3-μm latex beads. In sharp contrast to our results with 8-μm beads, neither of the two membrane markers cleared from the phagocytic cup (Figure 6, B–D). Indeed, both markers could be easily detected continuously on the perimeter of completely sealed phagosomes. The difference in membrane clearance between 3- and 8-μm beads was seen with multiple membrane markers and was highly significant ($p < 0.005$ for all; see Figure 6D). The differential behavior of the two types of beads was most apparent when cells were exposed simultaneously to both 3- and 8-μm particles. As illustrated in Figure 6, A and D, when the plasmalemma was prelabeled with fluorescent cholera toxin B subunit, clearance of the marker from the phagocytic cup was much more pronounced in the case of the large beads.

During Fcγ-receptor mediated phagocytosis both VAMP3 and VAMP7 have been shown to be recruited to the phagocytic cup (Bajno *et al.*, 2000; Braun *et al.*, 2004; Murray *et al.*, 2005). Using GFP-tagged VAMP3 and VAMP7 transfected into RAW macrophages, we confirmed that recruitment of these SNAREs also occurs when 8-μm beads are ingested (data not shown). We next investigated whether markers of additional endomembrane compartments were also delivered to large phagosomal cups. Given the longer time required for large particles to be internalized, we considered whether the early stages of phagosomal maturation—normally observed after complete internalization of the parti-

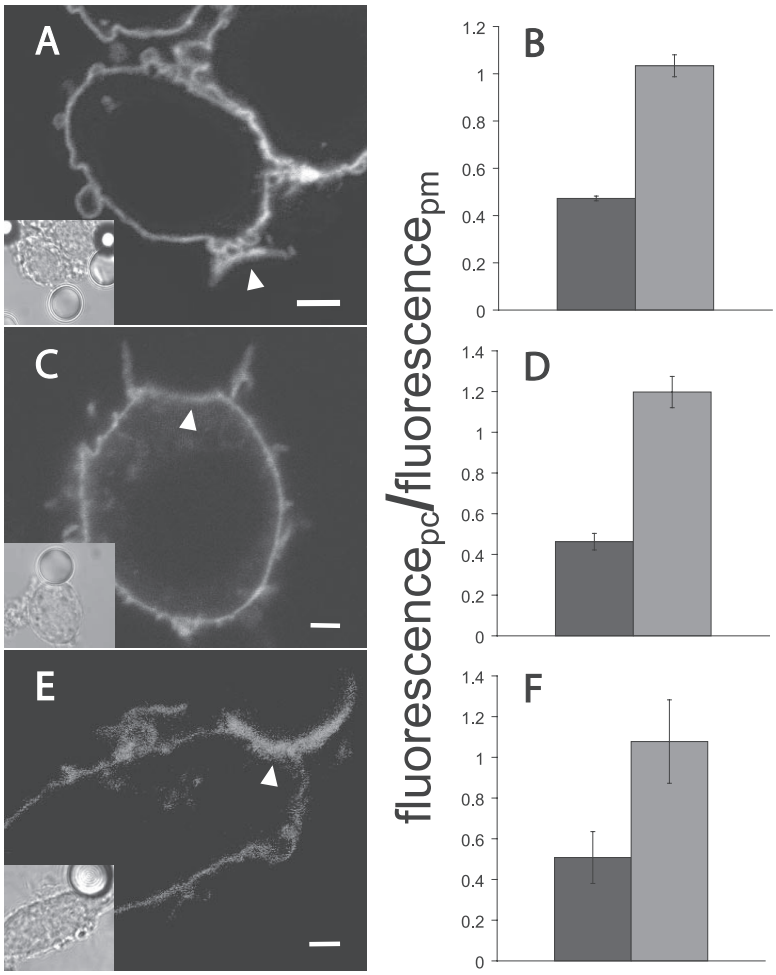

Figure 5. Inhibition of exocytosis prevents clearance of membrane markers. (A) YFP-GPI–transfected RAW cells were pretreated with 10 μM colchicine before exposure to 8-μm opsonized beads. Representative fluorescence (main panel) and DIC images (inset) are shown. (B) Quantitation of the fluorescence intensity at the base of the phagocytic cup (pc) relative to that of the plasma membrane (pm) in control (■) versus colchicine-treated cells (▨). (C) YFP-GPI–transfected RAW cells were pretreated with 100 μM LY294002 before exposure to 8-μm beads. (D) Quantitation of the fluorescence intensity at the base of the phagocytic cup relative to the plasma membrane in control (■) versus LY294002-treated cells (▨). (E) YFP-GPI-expressing RAW cells were cotransfected with DN-NSF and RFP at a 9:1 ratio. Transfected cells were identified by their red fluorescence. One such cell is illustrated. (F) Quantitation of the fluorescence intensity at the base of the phagocytic cup relative to the plasma membrane in cells transfected with either DN-NSF (▨) or with WT-NSF (■). Data in B, D, and F are means ± SE of over 30 determinations. For all comparisons, $p < 0.05$. Insets show corresponding DIC images. Scale bar, 2 μm.

cle—were initiated concomitantly with engulfment, possibly contributing to the clearance of plasmalemmal markers. GFP-tagged forms of the early endosomal markers Rab5 and EEA1 were transfected into RAW macrophages, and their distribution was monitored during phagocytosis of 8-μm beads. We were unable to detect any accumulation of Rab5 or EEA1 at the phagocytic cup in live cells (see Supplementary Figure S1). Using an analogous approach, we screened for the recruitment of late endosomes, lysosomes, and the endoplasmic reticulum to the phagocytic cup of large beads (Supplementary Figure S1). None of these markers accumulated noticeably at the cup. The data therefore suggest that the pronounced clearance of the surface markers from the base of large phagocytic cups is either due to the more effective delivery of endomembranes from the same sources detected earlier (Bajno *et al.*, 2000; Braun *et al.*, 2004) or to the contribution of a specialized compartment that remains to be identified.

Inhibition of Exocytosis Reduces Phagocytosis Preferentially of Large Particles

Delivery of endomembranes during the course of phagocytosis is likely intended to enable particle engulfment, while preserving the cellular surface-to-volume ratio. Given that inhibition of microtubules or of NSF function prevented exocytosis to the cup (as measured by lack of clearance of membrane markers), we postulated that particle internalization should be significantly attenuated under these conditions. In particular, internalization of large particles is expected to be preferentially affected, as these should require more endomembranes for engulfment than would smaller particles (Cox *et al.*, 1999). To validate this premise, RAW macrophages were pretreated with 10 μM colchicine for 10 min before allowing them to ingest either 3- or 8-μm beads. As predicted, the microtubule-dissociating drug attenuated phagocytosis of the larger particles significantly ($p < 0.05$) compared with the ingestion of the smaller beads (Figure 7, ■). Similarly, the inhibition induced by NSF was more profound for large than for small beads (Figure 7, ▨, $p < 0.05$).

Turnover of the Membrane Spares the Fcγ Receptor But Not Its Effectors

The extensive remodeling of the membrane at the phagocytic cup could have profound effects on the phagocytic signaling apparatus, selectively retaining some components while depleting others. To examine this possibility, we compared the distribution of phagocytic receptors with that of generic membrane markers at the cup. We chose to study FcγRIIA because, unlike other Fcγ receptor species, it consists of a single polypeptidic chain and does not require ancillary subunits (Gessner *et al.*, 1998). This is technically advantageous, because transfection of a single GFP-tagged construct suffices for analysis of receptor distribution. The ectopically expressed receptor was located

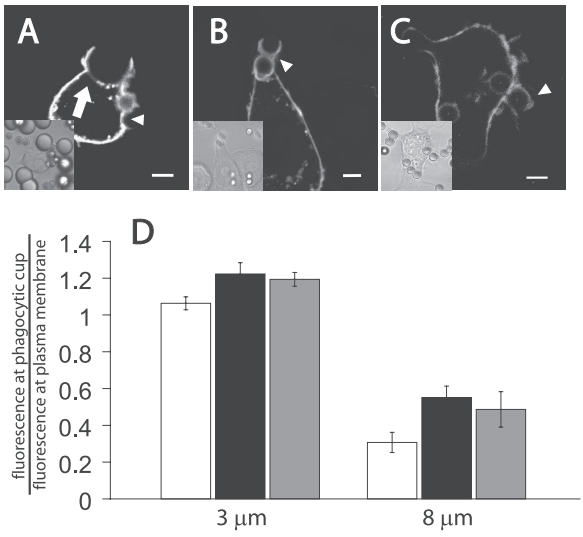

Figure 6. Clearance of membrane markers is size-dependent. (A) RAW cells were labeled with Alexa 555–conjugated cholera toxin B subunit before simultaneous exposure to 3- and 8-μm opsonized beads. (B) Cells transfected with YFP-GT46 exposed to 3-μm beads. (C) Cells transfected with YFP-GPI exposed to 3-μm beads. Insets show corresponding DIC images. Scale bar, 3 μm. (D) Quantitation of the fluorescence intensity at the base of the phagocytic cup relative to the plasma membrane, measured 1 min after exposure to the either small (3 μm) or large (8 μm) beads, as indicated. Cells labeled with cholera toxin (□), YFP-GT46 (■), or YFP-GPI (▨) are shown. Data in D are means ± SE of over 30 determinations. The differences between small and large beads were statistically significant ($p < 0.05$) for all markers.

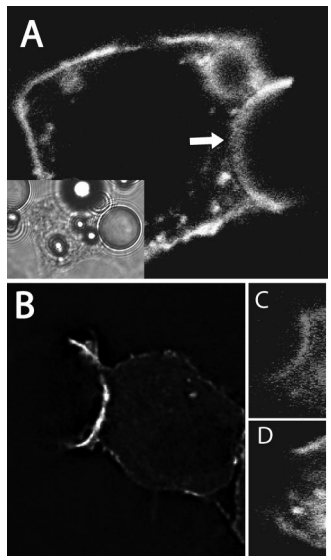

Figure 8. FcγRIIA receptors are selectively retained during remodeling. (A) RAW cells transfected with FcγRIIA-GFP were exposed simultaneously to 3- and 8-μm opsonized latex beads. A representative confocal image acquired 1 min after addition of particles is illustrated. Arrow indicates retention of the receptor at the base of a large phagocytic cup. Inset is corresponding DIC image. (B) Accumulation of FcγRIIA-GFP at the phagocytic cup. Enrichment of receptors at the cup, such as the one illustrated here, was noted in ≈30% of the cells. (C and D) RAW cells cotransfected with FcγRIIA-CFP and YFP-GT46 were allowed to internalize 8-μm beads. The distribution of the receptor (C) and of YFP-GT46 (D) during the course of internalization is illustrated.

largely in the plasmalemma (73 ± 4%), with the remainder found in endomembrane vesicles. FcγRIIA-GFP was readily detected at the phagocytic cup (Figure 8A). In fact, in a fraction of cells (≈30%) the receptor accumulated at the cup attaining densities that clearly surpassed that of the bulk, unengaged plasma membrane (Figure 8B). Remarkably, unlike the markers tested earlier, the receptors did not clear from the base of the cup as the particle was being internalized. To prove that membrane remodeling at the phagocytic cup preferentially spared Fcγ receptors, we cotransfected CFP-tagged FcγRIIA and YFP-tagged GT46. Although both FcγRIIA and GT46 are single-span transmembrane proteins, they behaved quite distinctly at the phagocytic cup. Although GT46 underwent extensive clearance from the cup well before the phagosome had sealed, FcγRIIA-CFP remained present (Figure 8, C and D). In other experiments, we expressed FcγRIIA-GFP and PM-RFP, a hydrophobically anchored marker of the inner leaflet of the plasma membrane. As seen for the transmembrane marker, GT46, the hydrophobic probe PM-RFP was depleted from the nascent phagocytic cup, whereas FcγRIIA-GFP remained (data not shown).

We considered the possibility that the persistence of FcγRIIA at the cup was due to ongoing delivery of intracellular receptors during the course of phagocytosis. To analyze this possibility, we selectively labeled the intracellular recycling pool of receptors using a Fab fragment obtained from a FcγRIIA-specific antibody (IV.3), coupled with a secondary Cy3-labeled Fab fragment. Label attached to exofacial receptors after the period allowed for internalization was then displaced by a rapid acid pulse, as described in *Materials and Methods*. The cells were next exposed to particles and the distribution of total receptors was studied by monitoring the fluorescence of GFP, whereas that of the internal recycling pool was followed monitoring the red fluorescence of Cy3. Although green fluorescence persisted at the cup, no delivery of previously internalized receptors was detectable in the membrane lining the bead (not illustrated). These observations suggest that FcγRIIA are not dynamically replaced by delivery of receptors stored in endomembranes, but that they are instead retained by interaction with their ligands on the particle surface.

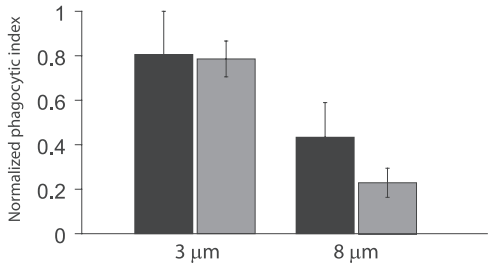

Figure 7. Inhibition of exocytosis attenuates phagocytosis in a size-dependent manner. YFP-GPI-transfected RAW cells were pretreated with 10 μM colchicine before exposure to 8- or 3-μm opsonized latex beads. Colchicine (■) reduced phagocytosis of 8-μm opsonized beads ($p < 0.05$) but had no significant effect on uptake of 3-μm beads. YFP-GPI–transfected RAW cells were cotransfected with either DN-NSF or WT-NSF and RFP in a 9:1 ratio (▨). Transfection with DN-NSF greatly inhibited phagocytosis of 8-μm beads ($p < 0.05$ relative to WT-NSF) with less effect on 3-μm beads.

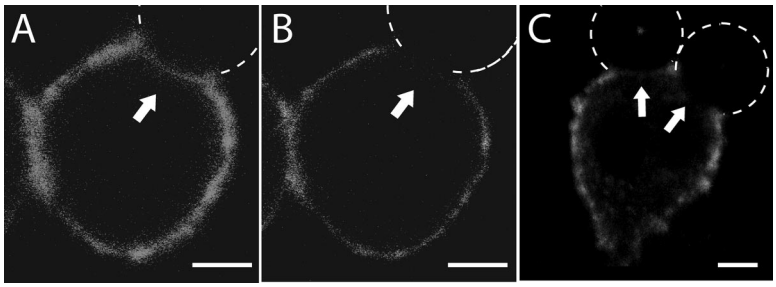

Figure 9. Differential depletion of FcγRIIA and Lyn from forming phagosomes. RAW cells were cotransfected with FcγRIIA-GFP (A) and Lyn-KD-YFP (B) and then allowed to ingest 8-μm opsonized beads, while monitoring fluorescence by confocal microscopy. A representative image acquired (using the LSM510 Meta to achieve spectral separation) ≈1 min after engagement of the bead is illustrated. In C untransfected cells were allowed to engage opsonized beads and were then fixed, permeabilized, and immunostained for endogenous Lyn. The dashed lines show the bead outlines. Arrows point to the base of the phagocytic cup. Scale bars, 4 μm.

Membrane Remodeling Results in Physical Separation of the Fc Receptor from Lyn

The first step in the signaling cascade triggered by Fcγ receptors involves activation of Src-family kinases such as Lyn, Hck, and Fgr (Fitzer-Attas *et al.*, 2000). These are targeted to the membrane by means of hydrophobic interactions and are not tightly attached to the receptors. We therefore wondered if the kinases remain attached to the receptors during phagosomal remodeling or whether they are displaced by the bulk membrane flow, which might terminate their signaling at the cup. We coexpressed FcγRIIA-GFP and Lyn tagged with YFP (Lyn-YFP) in RAW cells. Because heterologous expression of wild-type Lyn-YFP had toxic effects on the macrophages, a kinase-dead version of the enzyme (K275R) was used instead. The transfected RAW cells exhibited normal targeting to the plasma membrane of both constructs and were capable of phagocytosis of large beads. Importantly, we observed that although the Fcγ receptors remained at the cup during phagocytosis (Figure 9A), there was extensive clearance of the kinase (Figure 9B). To ensure that the ectopically expressed kinase-dead Lyn reflected the behavior of the WT kinase, we repeated the experiments in untransfected cells and analyzed the distribution of endogenous Lyn by immunostaining. As shown in Figure 9C, the endogenous kinase was also largely cleared from the membrane underlying the large particles during the advanced stages of cup formation.

DISCUSSION

The central finding from this study is that the membrane of the phagocytic cup is almost completely remodeled during the course of internalization of large but not small particles. The extensive depletion from the cup applied to multiple markers, but was much less pronounced for Fcγ receptors, which are anchored to ligands on the particle. This rules out optical artifacts as the explanation for clearance, which was also confirmed independently by labeling the particles (Figure 1). Remodelling of the phagocytic cup is due to insertion of endomembranes and results in the physical separation of Fcγ receptors from their most proximal and important effectors, the Src-family kinases. We also found that extensive, localized endocytosis occurs at the phagocytic cup in macrophages.

The mechanism responsible for cup remodeling was studied in detail, using a variety of markers and approaches. Selective removal of cup components by the activated endocytosis was considered, but was discounted based on the paucity of membrane markers in the endocytic vesicles and the persistence of clearance in cell transfected with dominant negative dynamin I. We attribute this observation to "kiss and run" events where some of the endomembranes undergo only transient fusion with the plasmalemma, allowing uptake of the extracellular dye, but little intermixing with components of the cup membrane. We also considered whether clearance was caused by segregation of lipid microdomains such as rafts, wherein Fcγ receptors are proposed to partition after clustering (Kwiatkowska *et al.*, 2003). However, markers known to distribute selectively to rafts were displaced from the phagocytic cup at a rate and to an extent similar to those found for nonraft markers. In addition, we observed that constituents of the inner and outer monolayer were affected similarly, arguing against uncoupling of the two aspects of the membrane bilayer. Instead, using multiple independent methods, we concluded that exocytosis of endomembranes was largely responsible for membrane remodeling. Several lines of evidence buttress this conclusion. First, markers of endomembrane compartments, particularly VAMP-3, were found to appear at the phagocytic cup (Bajno *et al.*, 2000; Murray *et al.*, 2005 and data not shown). Second, the density of ferritin-labeled plasmalemmal components decreased in the membrane surrounding the particle. Third, conditions that inhibit exocytosis, such as expression of DN-NSF, precluded clearance of plasma membrane components from the cup. The greater need for insertion of endomembranes to completely surround the particles also explains why large beads are more susceptible to inhibition than their smaller counterparts. This applied to both DN-NSF and colchicine and parallels the differential sensitivity of beads of varying size to wortmannin, which likely impairs exocytosis at the phagocytic cup (Cox *et al.*, 1999). Macrophages may sense particle size by a combination of membrane curvature (Champion and Mitragotri, 2006), the number of receptors engaged, and the time required to complete phagosome sealing. Taken together, our data implicating exocytosis are in good agreement with earlier demonstrations that the membrane capacitance often increases during engulfment (Holevinsky and Nelson, 1998; Di *et al.*, 2003) and that the binding of styryl dyes increases when macrophages spread onto IgG-coated surfaces (Cox *et al.*, 1999).

Given the requirement for insertion of additional membrane to complete engulfment of large particles, the concomitant stimulation of endocytosis would appear paradoxical. Its role may be the selective retrieval of components required for additional rounds of exocytosis, as is believed to be the case at neuronal synapses after release of neurotransmitter-containing vesicles. On the other hand, endocytosis at the phagocytic cup could serve a specialized immune function, sampling antigens for presentation in a manner analogous to the pinocytic uptake performed by dendritic cells.

What are the functional consequences of the extensive turnover of the membrane at the phagocytic cup? Fcγ receptor-mediated signaling begins with recruitment of effector tyrosine kinases. Src-family kinases are initially activated and they, in turn, trigger recruitment of Syk and the activation of multiple signaling pathways, including stimulation

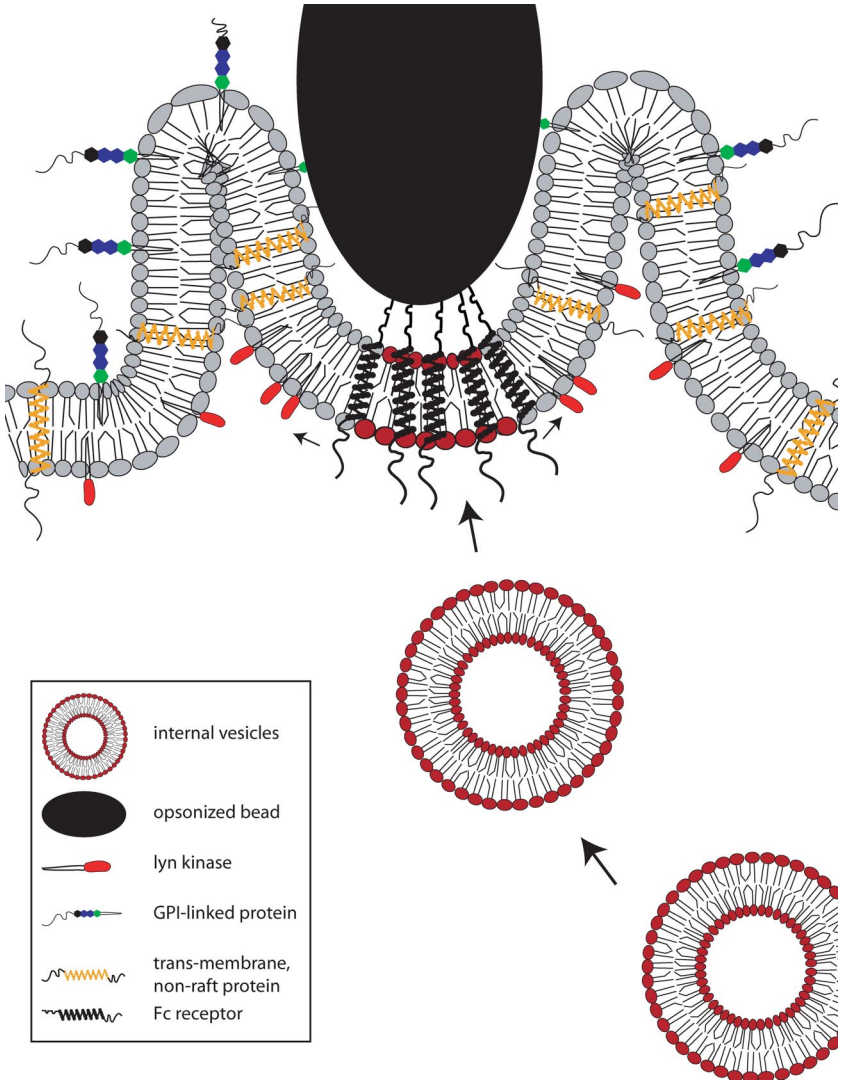

Figure 10. Schematic representation of membrane remodeling at the phagocytic cup. Focal insertion of endomembranes by exocytosis results in extensive turnover of the membrane at the base of the cup, including lipid raft and non-raft membrane constituents. This results in physical separation of the Fc receptors, which are immobilized by interaction with particulate ligands, from its membrane-associated effectors, such as Lyn kinase.

of various protein and lipid kinases as well as phospholipases and the engagement of multiple GTPases, culminating with cytoskeletal and membrane remodeling. The exocytic insertion of endomembranes must impact on this sequence, to the extent that the Fcγ receptors are physically separated from (the bulk of) the Src-kinases, as indicated by the redistribution of Lyn and of the PM-GFP probe, which is based on the motif that targets Src-family kinases to the membrane (Hof and Resh, 1997). It is most likely that exclusion of Lyn and other membrane-anchored kinases from the cup will contribute to termination of signaling, despite the retention of engaged receptors. Though the receptors are phosphorylated early during phagosome formation, active phosphatases such as SHP are recruited to the site (Strzelecka-Kiliszek *et al.*, 2002) and will likely eliminate phosphotyrosine residues rapidly if displacement of the kinases prevents continued phosphorylation. In this regard, it is noteworthy that following the initial burst of polymerization, actin disassembles from the cup with a time course and spatial distribution that closely parallel the remodeling of the membrane illustrated here (see Supplementary Movie 1).

In summary, our findings suggest a new mode of termination of signaling, mediated by the physical separation between receptors and their effectors and caused by massive delivery of endomembranes to sites of activation (Figure 10). The accompanying changes in phospholipid composition of the membrane are also likely to contribute to signal termination, since the phosphoinositide content of endomembranes differs markedly from that of the plasmalemma.

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